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APPLICATION

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TITLE:	METHOD FOR IDENTIFICATION AND DETECTION OF MICROORGANISMS USING GYRASE GENE AS AN INDICATOR
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METHOD FOR IDENTIFICATION AND DETECTION OF MICROORGANISMS USING GYRASE GENE AS AN INDICATOR

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention is involved in a method for the identification and detection of organisms using the sequences of their genes encoding the B subunit of the DNA gyrase.

This invention is useful in medical fields as well as various industrial fields where the identification/classification or detection/monitoring of specific microorganisms (bacteria, yeasts, fungi, archaea and bacteria), especially bacteria, is necessary.

2. Prior Art

Conventionally, the identification/classification of living organisms has been carried out using the combination of biochemical and morphological tests. However, these tests often did not provide unequivocal answers to the taxonomic positions of tested organisms.

Recently, the taxonomy of organisms, in particular of bacteria, using rRNA sequences became fashionable. There are many reasons why rRNA molecules have been selected as standard molecules for the molecular taxonomy. They are constituents of all organisms. They exist in abundance, and therefore, can readily be isolated and characterized. For sequence comparison, many conserved regions of rRNA molecules allowed the alignment between distantly related organisms, while variable regions are useful for the distinction of closely related organisms (van de Peer, Y., S. Chapelles, and R. de Wacher. 1996. A quantitative map of nucleotide substitution rates in bacterial rRNA. Nucleic Acids Res. 24: 3381-3391; and Gutell, R.

R., N. Larsen, and C. R. Woese. 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. Microbiol. Rev. 58: 10-26). Furthermore, there is a few evidence for the horizontal transfer of rRNA genes although many other genes are expected to have frequently been transferred from one species to other distantly related species. At present, rRNA sequences are accumulating rapidly and they are accessible via an international database (Ribosomal Database project, http://rdp.life.uiuc.edu/).

However, as is clear from the fact that the evolution speed of rRNA genes is extremely slow, there is little difference in the rRNA sequences between closely related organisms. Therefore, in many times, species belonging to the same genus could not be discriminated by the analysis using rRNA sequences. For example, it is said that bacteria sharing more than 97 % of identity in their 16S rRNA sequences (bacterial small subunit rRNA) might belong to the same species. However, there are cases of bacteria exhibiting more than 99 % identity in their 16S rRNA sequences, and yet belonging to two distinct species as revealed from DNA hybridization analysis. Evidently, due to the slow speed of divergent evolution of the 16S rRNA gene, the resolution of 16S rRNA-based analysis between closely related organisms is lower than that of DNA hybridization analysis (Stackebrandt, E. and Goebel, B. M. 1994. Taxonomic note: a place far DNA-DNA reassociation and 16S rRNA sequence analysis in the present species difinition in bacteriology. *Int. J. Syst. Bacteriol. 37: 463-464*).

Other problems exist in the rRNA-based phylogenetic analysis. To establish a phylogenetic relationship based on rRNA sequences, these sequences should be aligned. The alignment of rRNA sequences composed from four different constituents (AUCG), however, is not easy, and requires some expertise. The correct sequencing of rRNA genes is also difficult largely due to their highly ordered structure. Furthermore, polymorphism of rRNA was found in some organisms.

In contrast, protein-encoding genes have evolved more rapidly than rRNA-encoding genes, since they allow the so-called neutral mutations that do not cause any amino acid substitutions in their gene products. It is then expected that, by using such protein-encoding genes, more precise phylogenetic analysis can be performed than by using rRNA sequences. Thus, the present inventors have developed and applied a method for the identification/classification or detection/monitoring of organisms using the sequences of *gyr B* genes encoding the B subunit of DNA gyrases (Yamamoto, S. and Harayama, S. 1995. PCR Amplification and Direct Sequencing of *gyr B* Genes with Universal Primers and Their Application to the Detection and Taxonomic Analysis of *Pseudomonas putida* Strains. *Appl. Environ. Microbiol.* 61: 1104-1109; Yamamoto, S. and Harayama, S. 1996. Phylogenetic Analysis of *Acinetobacter* Strains Based on the Nucleotide Sequences of *gyr B* Genes and on the Amino acid Sequences of Their Products. *Int. J. Syst Bacteriol.* 46: 506-511; Yamamoto, S. and Harayama S. 1998. Phylogenetic relationships of *Pseudomonas putida* strains deduced from the nucleotide sequences of *gyr B*, *rpoD* and 16S rRNA genes. *Int. J. Syst Bacteriol.* 48: 813-819; Yamamoto S., Bouvet P. J. M. & Harayama, S. 1998. Phylogenetic structures of the genus *Acinetobacter* based on the *gyr B* sequences: Comparison with the grouping by DNA-DNA hybridization. *Int. J. Syst. Bacteriol.* (in press); Harayama, S. and Yamamoto, S. 1996. P hylogenetic Identification of Pseudomonas Strains Based on a Comparison of *gyr B* and *rpoD* Sequences. p. 250-258 in Molecular Biology of Pseudomonads, edited by T, Nakazawa, K. Furukawa, D Haas, S. Silver. ASM Press, Washington, D. C.: and Watanabe, K., Yamamoto, S., Hino, S. and Harayama, S. 1998. Population dynamics of phenol-degrading bacteria in activated sludge determined by *gyr B*-targeted quantitative PCR. *Appl. Environ. Microbiol.* 64: 1203-1209).

DNA topoisomerases are essential for the replication, transcription, recombination and repair of DNA and control the level of supercoiling of DNA molecules by cleaving and resealing the phosphodiester bond of DNA. They are classified into type I (EC 5.99. 1.2) and type II (EC 5.99.1.3) according to their enzymatic properties. The DNA

gyrase is a type II topoisomerase that is capable of introducing negative supercoiling into a relaxed closed circular DNA molecule. This reaction is coupled with ATP hydrolysis. DNA gyrase can also relax supercoiled DNA without ATP hydrolysis. DNA gyrase consists of two subunit proteins in the quaternary structure of A2B2. The A subunit (GyrA) has a molecular weight of approximately 100 kDa while the B subunit (GyrB) has a molecular weight of either 90 kDa or 70 kDa (Wigley, D. B. 1995. Structure and mechanism of DNA topoisomerases. *Ann. Rev. Biomol. Struct.* 24: 185-208). The genes for DNA gyrase or its isofunctional enzymes should exist in all organisms as they are indispensable for the cell proliferation.

As described above, the present Inventors have already developed and applied successfully the method for the identification/classification or detection/monitoring of organisms using *gyr B* sequences. In this method, a *gyr B* gene fragment of an organism of interest is amplified by PCR using primers designed from the two amino acid sequences, His-Ala-Gly-Gly-Lys-Phe-Asp and Met-Thr-Asp-Ala-Asp-Val-Asp-Gly, which are highly conserved among the GyrB sequences of many organisms. Subsequently, the amplified fragments are subjected to direct sequencing. Since the *gyr B* genes code for proteins, they have frequently undergone neutral mutations. Thus, the nucleotide sequence of the *gyr B* genes vary considerable even among related organisms. For this reason, the above method has been shown to be effective for discriminating organisms at a level of species of subspecies.

The above-mentioned PCR primers designed from the highly conserved amino acid sequences of GyrB were effective in many but not all bacterial species for the PCR amplification of *gyr B*. From DNA of some bacterial species, no PCR amplification was observed using these primers.

Besides, there was another problem associated with these primers. The genes for type IV topoisomerese (ParE) were also amplified from DNA of some bacterial species

by using these primers. Topoisomerase IV (ParE) is a bacterial enzyme that appears to be closely related to DNA gyrase. This enzyme involves in the partition of chromosomes into daughter cells. If a ParE gene but not *gyr B* gene is amplified from a DNA, and if a phylogenetic analysis is carried out without recognizing that the amplified sequence is *parE* but not *gyr B*, it will bring some confusion to the phylogenetic analysis. To avoid such problem associated with the amplification of paralogous genes, primers which do not amplify ParE should be developed.

OBJECTIVE AND SUMMARY OF THE INVENTION

It is an object of the present invention to solve the above-described problems of the primers and to provide a means which enables the identification/classification and detection/monitoring of a wide range of organisms using *gyr B* sequences.

Comparing the amino acid sequence data of GyrB collected by the inventors with those of ParE, the inventors have found that a *gyrB* gene DNA fragment only can be isolated from a wide range of microorganisms by performing PCR using two specific primers (forward and reverse primers), thereby completing the present invention.

First, the present invention relates to a method for the indentification and detection of a microorganism, comprising the following steps (1) to (5):

(1) synthesizing two specific primers,

(2) amplifying *gyrB* gene DNA from the microorganism using the above two primers to produce a *gyrB* gene DNA fragment,

(3) isolating the above DNA fragment,

(4) determining the nucleotide sequence of the above DNA fragment, and

(5) identifying and detecting the microorganism by comparing the nucleotide sequence of the amplified *gyrB* gene DNA fragment to known *gyrB* gene DNA fragment sequences.

Second, the present invention relates to a method for the identification and detection of a microorganism, comprising the following steps (1) to (7):

(1) synthesizing two specific primers,

(2) amplifying *gyrB* gene DNA from the microorganism using the above two primers to produce a *gyrB* gene DNA fragment,

(3) synthesizing two pairs of specific primers,

(4) amplifying the *gyrB* gene DNA fragment produced in step (2) using the above two pairs of primers to produce two *gyrB* gene DNA fragments,

(5) isolating the above two DNA fragments,

(6) determining the nucleotide sequences of the above two DNA fragments, and

(7) identifying and detecting the microorganism by comparing the nucleotide sequences of the above two *gyrB* gene DNA fragments to known *gyrB* gene DNA fragment sequences.

Third, the present invention relates to a method for the indentification and detection of a microorganism, comprising the following steps (1) to (5):

(1) synthesizing two pairs of specific primers,

(2) amplifying *gyrB* gene DNA from the microorganism using the above two pairs of primers to produce *gyrB* gene DNA fragments,

(3) isolating the above two DNA fragments,

(4) determining the nucleotide sequences of the above two DNA fragments, and

(5) identifying the microorganism by comparing the nucleotide sequences of the above two *gyrB* gene DNA fragments to known *gyrB* gene DNA fragment sequences.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the locational relationship between the amino acid sequence (a) through (l) and the amino acid sequences of GyrB of several organisms.

DETAILED DESCRIPTION OF THE INVENTION

Hereinbelow, the present invention will be described in detail.

The method for identification and detection of a microorganism of the present invention is a method for identifying and detecting of a microorganism based on the nucleotide sequence of a *gyrB* gene. The nucleotide sequence data of a *gyrB* gene may be obtained from a DNA fragment amplified by PCR (one fragment method), or may be obtained from two fragments having a mutually overlapping portion (two fragments method).

In order to identify and detect a microorganism with high accuracy, a nucleotide sequence of approx. 1kb DNA fragment is required. In 1994, Yamamoto and Harayama filed a patent application for an invention relating to primers to amplify a *gyrB* fragment, which is a gene encoding the B subunit of DNA gyrases (Japanese Patent Application Laid-Open (Kokai) No. 1995-213299). However, the subsequent study has clarified that, according to the disclosure of the above application alone, the identification and detection of a microorganism may be difficult for the following reasons:

(1) It is difficult to determine a nucleotide sequence by a one-time sequencing reaction.

(2) Some strains are inefficiently amplified with only the known primer sequences.

(3) In some cases, it is difficult to determine a nucleotide sequence, since *parE*, being a homologous gene of *gyrB*, is amplified together with *gyrB*. In other cases, an incorrect identification of a microorganism is performed, since *parE* is selectively amplified.

To overcome the above-stated problems, in addition to the primer sequences disclosed by Yamamoto and Harayama in 1995, the present invention provides: primer sequences conserved among *gyrB* genes of a large number of bacteria; primer sequences specific for *gyrB* genes, not appearing in *parE* genes, or primer sequences appearing in

both *gyrB* and *parE* genes, which allow easy distinction of an amplified *parE* fragment from an amplified *gyrB* fragment; and a method for specific amplification of a *gyrB* gene by the combined use of the above primer sequences.

To overcome the above-stated problems, sometimes it may be more preferable to amplify separately two DNA fragments having a mutually overlapping portion, and to determine the nucleotide sequence of each fragment, followed by connecting the obtained two sequences before using the data.

In order to amplify the two fragments, DNA obtained from the microorganism (which also contains various DNAs as well as *gyrB* gene DNA) may be used as is, as a template ("one step – two fragments method"), but as another option, after performing PCR using primers capable of specifically amplifying a *gyrB* gene DNA, the obtained amplified products may be used as a template ("two steps – two fragments method").

Primers used for the above three types of DNA amplification methods ("one fragment method", "one step – two fragments method" and "two steps – two fragments method") are shown in Figure 1. The figure shows the amino acid sequence of each GyrB of 3 kinds of strains, *Bacillus subtilis* 168, *Escherichia coli* K-12 and *Pseudomonas putida* PRS2000, and (a) – (l) in the figure respectively correspond to amino acid sequences (a) – (l) shown as follows:

(a) (Pro or Ser)-(Ala or Thr)-(Ala, Val or Leu)-Glu or Asp)-(Val or Thr)-(Ile or Val)-(Met, Leu or Phe)-Thr-(Val, Gln or Ile)-Leu-His-Ala-Gly-Gly-Lys-Phe-(Asp or Gly)-(Asp, Gly, Asn or Ser)-(Ser, Lys, Gly, Asp or Asn) [SEQ ID NO.69]

(b) Gly-Gly-Thr-His [SEQ ID NO.70]

(c) (Ile or Leu)-Met-Thr-Asp-Ala-Asp-Val-Asp-Gly-(Ala or Ser)-His-Ile-Arg-Thr-Leu [SEQ ID NO.71]

(d) Arg-Lys-Arg-Pro-(Gly or Ala)-Met-Tyr-Ile-Gly-(Ser or Asp)-Thr [SEQ ID NO.72]

(e) Gln-(Thr or Pro)-(Lys or Asn)-(Thr, Asp, Gly, Lys, Ser, Phe or Tyr)-Lys-Leu [SEQ ID NO.73]

(f) (Tyr or Phe)-Lys-Gly-Leu-Gly-Glu-Met-Asn-(Ala or Pro) [SEQ ID NO.74]

(g) Val-Glu-Gly-Asp-Ser-Ala-Gly-Gly-Ser [SEQ ID NO.75]

(h) Lys-(His or Val)-Pro-Asp-Pro-(Gln or Lys)-Phe [SEQ ID NO.76]

(i) Leu-Pro-Gly-Lys-Leu-Ala-Asp-Cys-(Ser or Gln)-(Ser or Glu)-(Lys or Arg)-Asp-Pro-(Ala or Ser) [SEQ ID NO.77]

(j) Gln-Leu-(Trp or Arg)-(Glu or Asp)-Thr-Thr-(Met or Leu)-(Asp or Asn)-Pro [SEQ ID NO.78]

(k) Ala-(Lys or Arg)-(Lys or Arg)-Ala-Arg-Glu [SEQ ID NO.79]

(l) Phe-Thr-Asn-Asn-Ile-(Pro or Asn)-(Thr or Gln) [SEQ ID NO.80].

Two primers used for the "one fragment method" include the ones which are synthesized based on a single pair of amino acid sequences selected from the group consisting of sequence pairs (a) and (f), (a) and (j), (d) and (c), (d) and (f), and (d) and (j). The *gyrB* gene of members of *proteobacteria* has an approx. 500bp insertion sequence located between a sequence pair (c) and (f), and another sequence pair (c) and (j), on the other hand, *parE* which is a homologous gene thereof does not have such sequence (Kato, J.-i., Nishimura, Y., Imamura, R., Niki, H., Hiraga, S., and Suzuki, H. *New topoisomerase essential for chromosome segregation in E. coli Cell* 63, 393-404 (1990)). Accordingly, in a case where two primers synthesized based on amino acid sequence pairs (a) and (f), (a) and (j), (d) and (f), or (d) and (j) are used for identification and detection of a microorganism belonging to *proteobacteria*, DNA fragments of *gyrB* genes can easily be separated from those of *parE* genes by electrophoresis and so on, since the length of the amplified DNA fragments of the two types of genes is different.

Two primers used for the "one step – two fragments method" include the following amino acid sequence combinations:

	Amplified fragment 1	Amplified fragment 2

Combination 1	sequences (d) and (e)	sequences (h) and (c), sequences (h) and (f), or sequences (h) and (g)
Combination 2	sequences (a) and (i)	sequences (k) and (c), sequences (k) and (f), or sequences (k) and (g)
Combination 3	sequences (a) and (i)	sequences (l) and (c), sequences (l) and (f), or sequences (l)and (g)

Among amino acid sequences in the above table, sequences (i), (e), (h), (k) and (l) do not exist in ParE, and in many cases, are specific for GyrB. For example, these sequences are determined to be specific for GyrB in the strains shown in the following table.

Sequence	Strain
(i)	*Leclercia adecarboxylata* GTC 1267
	Pseudoalteromonas sp. A316
	Gordonia amarae DSM 46078
	Rhodococcus koreensis JCM 10743
	Shigella dysenteriae GTC 786
	Salmonella typhi P1
	Sphingomonas sp. MBIC 5538
	Serratia ficaria GTC 343
	Alteromonas macleodii MBIC 1375
	Vibrio fluvialis GTC 315
(e)	*Mycobacterium tuberculosis* KPM T21
	Bacteroides fragilis
	Acholeplasma laidlawii PG-8B
	Bacillus cereus JCM 2152
	Treponema denticola ATCC 35405
	Streptococcus pneumoniae 7785
	Arthrobacter oxidans IFO 12138
	Porphyromonas asaccharolytica JCM 6326
	Myxococcus xanthus ER-15
	Streptomyces coelicolor A3 (2)
(h)	*Pseudomonas putida* MBIC 5295
	Pseudoalteromonas sp. MBIC 3307
	Vibrio hollisae 89A 1962
	Aeromonas hydrophila P3
	Photobacterium histaminum JCM 8968
	Escherichia coli W3110

	Bacillus anthracis Pasteur #2 *Streptococcus pneumoniae* 7785 *Acinetobacter calcoacelicus* ATCC 23055 *Acholeplasma laidlawii* PG-8B
(k)	*Pseudomonas putida* MBIC 5295 *Pseudoalteromonas* sp. MBIC 3307 *Vibrio hollisae* 89A 1962 *Citrobacter* sp. MAM-1 *Comamonas terrigena* IAM 12052 *Salmonella typhimurium* *Acinetobacter junii* SEIP 14. 81 *Legionella pneumophila* ATCC 33152 *Escherichia coli* W3110 *Photobacterium histaminum* JCM 8968
(l)	*Leclercia adecarboxylata* GTC 1267 *Pseudoalteromonas* sp. A316 *Pseudomonas* sp. MBIC 5390 *Marinobacter* sp. MBIC 4911 *Shigella dysenteriae* GTC 786 *Salmonella typhi* P1 *Sphingomonas* sp. MBIC 5538 *Caulobacter* sp. GTC 1043 *Sinorhizobium fredi* ATCC 35423 *Comamonas* sp. GTC 866

Accordingly, by performing PCR with the combined use of the above primers, the *gyrB* gene DNA only can be specifically amplified.

Two primers used for the "two steps – two fragments method" include the

following amino acid sequence combinations:

	1st amplification step	2nd amplification step	
		fragment 1	fragment 2
Combination 1	sequences (a) and (c)	sequences (a) and (e)	sequences (b) and (c)
Combination 2	sequences (a) and (f)	sequences (a) and (e)	sequences (b) and (f)
Combination 3	sequences (d) and (f)	sequences (d) and (e)	sequences (b) and (f)

Among amino acid sequences in the above table, sequence (b) exists in GyrB of all known bacteria, and so primers synthesized based on this sequence can be applied for a wide range of microorganisms. Since sequence (b) consists of 4 amino acid residues, when PCR is performed using, as a template, DNA obtained from the microorganism with primers synthesized based on the sequence, there is a possibility to amplify DNA fragments totally irrelevant to *gyrB* genes. However, for the "two steps - two fragments method", amplified *gyrB* gene DNA fragments are used as a template, and so the above non-specific amplification can be avoided.

Since sequence (f) is conserved in GyrB genes of many bacteria, primers synthesized based on this sequence can be applied for a wide range of microorganisms. Since sequence (f) exists also in ParE, when PCR is performed using this sequence, not only *gyrB* gene DNA fragments, but also *parE* gene DNA fragments are amplified. However, the amplification of *parE* gene DNA fragments can be prevented by performing PCR using primers synthesized based on sequence (e) specific for GyrB.

"Primers synthesized based on sequences (a) – (l)" mean primers encoding all or a part of amino acid sequences (a) – (l), and having a length sufficient to specifically hybridize to a specific site of a template DNA.

The nucleotide sequences of primers synthesized based on sequences (a) – (l)

and amino acid sequences encoded by these nucleotide sequences are shown in the following table:

Sequence	Amino acid sequence	Nucleotide sequence
(a)	SEQ ID NO: 26	SEQ ID NO: 25
	SEQ ID NO: 30	SEQ ID NO: 29
	SEQ ID NO: 54, 55, 56, 57	SEQ ID NO: 53
(b)	SEQ ID NO: 34	SEQ ID NO: 33
	SEQ ID NO: 36, 37	SEQ ID NO: 35
(c)	SEQ ID NO: 28	SEQ ID NO: 27
	SEQ ID NO: 32	SEQ ID NO: 31
	SEQ ID NO: 42	SEQ ID NO: 41
(d)	SEQ ID NO: 46, 47	SEQ ID NO: 45
(e)	SEQ ID NO: 39, 40	SEQ ID NO: 38
(f)	SEQ ID NO: 44	SEQ ID NO: 43
(g)	SEQ ID NO: 49	SEQ ID NO: 48
(h)	SEQ ID NO: 63, 64	SEQ ID NO: 62
(i)	SEQ ID NO: 59	SEQ ID NO: 58
(j)	SEQ ID NO: 66, 67, 68	SEQ ID NO: 65
(k)	SEQ ID NO: 51, 52	SEQ ID NO: 50
(l)	SEQ ID NO: 61	SEQ ID NO: 60

Microorganisms applicable to the identification and detection method of the present invention include bacteria, yeast, Fungus, archaebacteria and the like.

PREFERRED EMBODIMENTS OF THE INVENTION

EXAMPLE 1

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 25 and 27 (corresponding to the amino acid sequences of SEQ ID NOS: 26 and 28, respectively) as primers and DNA from *Bacteroides vulgatus* IFO 14291 strain as a template. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 1 and 2, respectively. The PCR amplification conditions were as described below.

PCR amplification conditions:

96 ℃ 1 min;	48℃ 1 min;	72℃ 2 min:	3 cycles	
96 ℃ 1 min;	48℃ 1 min;	72℃ 2 min:	3 cycles	
96 ℃ 1 min;	48℃ 1 min;	72℃ 2 min:	30 cycles	
			Total: 36 cycles	

Primer concentration	1 μM each
dATP	200 μM each
Template DNA	< 1 μg/100 μl

AmpliTaq™ and the supplied PCR Buffer (Perkin Elmer) were used.

EXAMPLE 2

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 29 and 31 (corresponding to the amino acid sequences of SEQ ID NOS: 30 and 32, respectively) as primers and DNA from *Mycobacterium simiae* KPM 1403 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 3 and 4, respectively.

EXAMPLE 3

A PCR was performed using oligonucleotides represented by the nucleotide

sequences shown in SEQ ID NOS: 33 and 27 (corresponding to the amino acid sequences of SEQ ID NOS: 34 and 28, respectively) as primers and DNA from *Chitinophaga pinensis* DSM 2588 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 5 and 6, respectively.

EXAMPLE 4

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 25 and 35 (corresponding to the amino acid sequences of SEQ ID NO: 26 and SEQ ID NO: 36 or 37, respectively) as primers and DNA from *Flavobacterium aquatile* IAM 12316 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 7 and 8, respectively.

EXAMPLE 5

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 29 and 38 (corresponding to the amino acid sequences of SEQ ID NO: 30 and SEQ ID NO: 39 or 40, respectively) as primers and DNA from *Mycobacterium asiaticum* ATCC 25274 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 9 and 10, respectively.

EXAMPLE 6

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 41 and 43 (corresponding to the amino acid sequences of SEQ ID NOS: 42 and 44, respectively) as primers and DNA from

Cytophaga lytica IFO 16020 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 11 and 12, respectively.

EXAMPLE 7

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 45 and 48 (corresponding to the amino acid sequences of SEQ ID NO: 46 or 47 and SEQ ID NO: 49, respectively) as primers and DNA from *Synechococcus* sp. PCC 6301 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 13 and 14, respectively.

EXAMPLE 8

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 53 and 62 (corresponding to the amino acid sequences of SEQ ID NO: 54, 55, 56 or 57 and SEQ ID NO: 63 or 64, respectively) as primers and DNA from *Caulobacter crescentus* ATCC 15252 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 15 and 16, respectively.

EXAMPLE 9

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 53 and 58 (corresponding to the amino acid sequences of SEQ ID NO: 54, 55, 56 or 57 and SEQ ID No: 59, respectively) as primers and DNA from *Pseudomonas putida* ATCC 17484 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of

the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 17 and 18, respectively.

EXAMPLE 10

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 65 and 50 (corresponding to the amino acid sequences of SEQ ID NO: 66, 67 or 68 and SEQ ID NO: 51 or 52, respectively) as primers and DNA from *Synechococcus* sp. PCC 6301 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 19 and 20, respectively.

EXAMPLE 11

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 60 and 31 (corresponding to the amino acid sequences of SEQ ID NOS: 61 and 32, respectively) as primers and DNA from *Caulobacter crescentus* ATCC 15252 strain as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 21 and 22, respectively.

EXAMPLE 12

A PCR was performed using oligonucleotides represented by the nucleotide sequences shown in SEQ ID NOS: 25 and 43 (corresponding to the amino acid sequences of SEQ ID NOS: 26 and 44, respectively) as primers and DNA from an unidentified strain MBIC 1544 as a template. The PCR amplification conditions were the same as in Example 1. The nucleotide sequence of the amplified DNA fragment and the amino acid sequence deduced therefrom are shown in SEQ ID NOS: 23 and 24, respectively.

This nucleotide sequence was compared with the nucleotide sequence database possessed by the applicant. As a result, the unidentified strain MBIC 1544 was identified as *Cytophaga lytica.*

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EFFECT OF THE INVENTION

With the nucleotide sequence of *gyr B* determined by the pre sent invention, it is possible to classify or identify an unidentified microorganism strain quickly and accurately. Besides, according to the present invention, PCR primers for monitoring a specific microorganism which are needed in risk assessment in various bioprocesses can be designed easily. Also, the present invention enables highly accurate monitoring of changes in mycelial tufts.